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ASSOCIATIONS BETWEEN THE GUT MICROBIOME AND TEMPERAMENT IN INFANCY AND EARLY CHILDHOOD – A SYSTEMATIC REVIEW

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Early life temperament predicts personality and risk of psychiatric and behavioral disorders later in life. Variation in temperament is influenced by biological and environmental factors. Research implicates a connection between the gut microbiome and physical as well as mental health conditions, creating a biological mechanism of interest. The gut microbiota is associated with brain regions that are linked to neurodevelopment and control of behavior. The aim of this study is to gather and analyze scientific evidence of associations between gut microbiota (GM) composition and diversity, and temperament in infancy and early childhood.

This study is a systematic review based on a literature search of the PubMed database. A total of 25 studies were identified, of which seven were included in the review following application of eligibility criteria.

The findings suggest an association between higher Surgency/Extraversion and a specific gut microbiota structure (beta diversity). Evidence supporting a link between GM alpha diversity and temperament was limited and warrants further research. On the taxonomic level, some interesting patterns emerged. Results implicate that an abundance of *Bifidobacteriaceae* may promote Surgency/Extraversion as well as Effortful Control/Regulation in the first year of life. Additionally, the families of *Lachnospiraceae*, *Ruminococcaceae* and *Dialister* of the phylum Bacillota, were found to influence Surgency/Extraversion and Negative Affectivity implicating a gut-brain connection mediated by their shared ability to produce butyrate, a short-chain fatty acid with known health benefits. Although these observations find support in literature, they remain tentative owing to the limited number of studies as well as the observed significant heterogeneity in methodology and study populations. Further research is needed to replicate results across different populations and timelines as well as to expand the scope of research to include functional aspects of the GM.

Asiasanat: Gastrointestinal Microbiome, Gut Microbiota, Gut-Brain Axis, Temperament

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1 INTRODUCTION

Psychiatric disorders are responsible for a greater share of disability in the developed countries than any other category of diseases (Reeves et al., 2011). Moreover, the global prevalence of mental health disorders in children and adolescents 6-18 years of age, is almost 14% (Polanczyk et al., 2015). As with physical health disorders, childhood behavioral problems as well as mental health conditions later in life have early life antecedents (Caspi et al., 1996). Identification of early childhood markers of later life behavioral and mental health problems enables the possibility to develop interventions and prevention measures targeted at modifiable early life antecedents (Alving-Jessep et al., 2022).

1.1 Early life temperament and later life mental health

Differences in behavior during early life can be assessed by utilizing the concept of temperament (M. K. Rothbart, 2007). Although a precise definition of temperament is subject to ongoing debate, there exists a consensus that temperament manifests from infancy onward, has a strong neurobiological or genetic basis and is relative consistent across situations and time (de Pauw & Mervielde, 2010). In the widely used temperament model developed by Rothbart and Derryberry, the concept of temperament is defined as "individual differences in reactivity and self-regulation" (M. Rothbart & Derryberry, 1981). These differences are assumed to have a constitutional basis, which means that they are largely determined by the responsiveness of underlying psychobiological processes of the individual. Elaborating on this, the authors define constitutional as "the relatively enduring biological makeup of the individual, influenced over time by the interaction of heredity, life experience, and maturation". (Rothbart, 1981). Reactivity in turn refers to the physiological excitability of neural systems, whereas self-regulation refers to the behavioral and neural processes functioning to modulate this underlying, involuntary reactivity (De Pauw & Mervielde, 2010). Individual differences in reactivity and self-regulation are measured with temperament dimensions/scales which in turn are divided into three composite scales and numerous subscales. According to Rothbart, three broad temperamental systems influential in the development of personality can be identified early in life (M. K. Rothbart & Putnam, 2002). These are: Surgency/Extraversion, Negative Affectivity and Effortful Control/Regulation. Based on Rothbart and Putnam's article (M. K. Rothbart & Putnam, 2002), Berdan et. al summarize key features of these main dimensions as follows:

> Surgency/Extraversion is characterized by high activity level, high-intensity pleasure seeking, low shyness, and impulsivity. Negative Affectivity is characterized by sadness, discomfort, frustration, fear, and difficulty to soothe ... Finally, Effortful Control encompasses inhibitory control, attentional focusing, low-intensity pleasure, and perceptual sensitivity…

The three main dimensions of temperament constitute the composite scales of temperament used in questionnaires based on Rothbart's model. The composite scales encompass numerous subscales that vary according to the age group being assessed, and each subscale loads onto one composite scale. (See e.g., Rothbart et al., 2000).

Several studies have found that early life temperament influences personality and mental health later in life (Abulizi et al., 2017; Clark et al., 1994; Forbes et al., 2017; M. K. Rothbart et al., 2000; Sayal et al., 2013; Slobodskaya & Kozlova, 2016). For instance, higher scores in the main temperament dimension of negative affectivity during infancy have been found in several studies to be associated with a risk for developing symptoms of depression (Compas et al., 2004) and anxiety (De Pauw & Mervielde, 2010) in later life. Lower early life Effortful Control has been repeatedly demonstrated to predict later life externalizing problems both cross-sectionally and longitudinally (Eisenberg et al., 1996, 2000, 2001; Olson et al., 2005). Moreover, whether assessed through parent, teacher, or observational means, measures of temperament traits have been shown to be predictors of personality, behavior, and risk of psychopathology in later childhood and adolescence (Muris & Ollendick, 2005). As such, temperament presents a target for identifying early life markers of later mental health conditions.

1.2 The gut microbiome and mental health

In biology, the term microbiome refers to all microorganisms, their collective genomes and signaling molecules within an environment, whereas the term microbiota only refers to the microbes themselves (Berg et al., 2020). In other words, microbiome is the metagenome of the microbiota. In this study, the abbreviation GM refers to microbiota unless otherwise stated. More specifically, the research addressed in this review is focused on the bacteria inhabiting the human gut, instead of e.g., fungi or viruses. The human gut is home to a densely populated microbial ecosystem and several studies have implicated a connection between the gut microbiome and physical as well as mental health conditions (Cryan et al., 2019). In animal studies, transplantation of fecal microbiota of either stressed or obese animals to control animals resulted in significant observed alteration of anxiety (Messaoudi et

al., 2011). Furthermore, transferring gut microbiota of humans diagnosed with depression to germfree rats resulted in behavioral and physiological attributes characteristic of depression in the animals, thus suggesting causality (Kelly et al., 2016). In humans, pre- and probiotic consumption has been observed to positively affect mood and symptoms of anxiety (Steenbergen et al., 2015). Microbes have been shown to regulate host metabolism and are critical for immune system development and function (Sampson & Mazmanian, 2015). Recent research also indicates that the gut microbes also impact neurodevelopment and control of behavior (e.g., Needham et al., 2022; Vuong et al., 2020; for reviews see e.g., Rogers et al., 2016; Sampson & Mazmanian, 2015). In children, gut bacterial colonization has been shown to be directly associated to the maturation of both the central nervous system (CNS) and enteric nervous system (ENS) (Barbara et al., 2005; Stilling et al., 2014). The gut microbiota is intricately connected with specific brain regions that are in turn linked to emotional stimuli and the development of cognition (Tillisch et al., 2013). This so-called microbiota-gut-brain (MGB) axis is bidirectional and operates via multiple signaling pathways. The MGB axis includes the central nervous system (CNS), the autonomic nervous system (ANS), the enteric nervous system (ENS), the neuro-endocrine (e.g., serotonin) and neuro-immune (e.g., cytokine) pathways, and the gut microbiome. (Cryan et al., 2019). Bacteria in the gastrointestinal (GI) tract can activate neural pathways and modulate plasticity-related serotonergic and GABAergic signaling systems in the central nervous system (Foster & McVey Neufeld, 2013). The MGB pathway is also proven to affect the hypothalamic-pituitary-adrenal axis (HPA) (Sudo et al., 2004). Dysregulation of the HPA axis is, in turn, associated with depressive episodes. (Foster & McVey Neufeld, 2013). The various signaling pathways and functional mechanisms of the MGB are subject to ongoing research.

The intestinal microbiota and nervous system go through rapid changes in parallel during early postnatal period. Therefore, the GM and brain are believed to share similar sensitive periods of development during infancy and extending at least up until the second year of life (Borre et al., 2014) and according to some scholars, possibly even into adolescence (Cowan et al., 2020). Sensitive periods in GM development include birth and the early postnatal period as well as the beginning of complementary feeding, occurring typically around 6 months of age (as per WHO recommendation) (Cowan et al., 2020). Maturation of the gut microbiome is thought to occur at approximately 31-46 months of age after which the GM tends to stabilize (Stewart et al., 2018). Sensitive developmental periods of the GM align with neurodevelopmental periods of plasticity of the CNS including sensory function, learning,

language, and memory (Cowan et al., 2020). Considering the known bidirectional connectivity of the MGB axis in context with the parallel plasticity of the CNS and sensitivity of the GM to changes in early life it seems plausible, that disturbances of the developing gut microbiota can influence neurodevelopment, thus potentially altering behavioral development trajectories and leading to psychopathology later in life (as stated by e.g., Clarke et al., 2014).

The gut microbiome can well be labelled a hot topic in the scientific community, with articles in PubMed including “gut microbiome” or “gut microbiota” in the title increasing from 58 articles in the year 2008 to 5400 articles in the year 2022. This is an increase of over 12 700% in published research papers per year. So far, the rapid growth of research has not shown signs of slowing down. One of the key topics underlying this expansion of research has been the relationship between gut microbiome and mental health (Christian, 2019). Taking all of this into account, research investigating the association between early life temperament and gut microbiome has been surprisingly sparse for long, especially considering the known long-lasting influence of childhood temperament on adult personality and psychopathology. Fortunately, recent years have seen an increase in articles on this subject, which in turn provides a novel possibility for a systematic review of the current research on this field.

The objective of this systematic review is to gather and synthetize current evidence regarding possible associations between the gut microbiome and temperament traits in early childhood.

2 METHODS

A systematic review was conducted according to the methods set out in the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA 2020) statement (Page et al., 2021).

2.1 Data sources

A search of the academic database PubMed was conducted in October 2022. Search terms were established related to the core concepts of microbiome (microbio*, microflora*, bacteria*, metagenome*), childhood (child*, infant*, infancy) and temperament (temperament*). Boolean operators were added resulting in the following search phrase:

> (microbio* OR microflora* OR bacteria* OR metagenome*) AND (child* OR infant* OR infancy) AND temperament*

The search was repeated in February 2023 to ensure inclusion of the most up-to-date articles.

2.2 Eligibility criteria

Eligibility criteria were negotiated and established by MK and AA. Eligible for inclusion were all studies reporting on the associations between gut microbiome composition and/or diversity and temperament in children under the age of 7 years. In temperament assessments, the age of 7 years is often considered a cutoff point between early and middle childhood; consequently, the measures of temperament observed change from measures of early childhood to measures of middle childhood (see e.g., Rothbart et al., 2001). To best serve the purpose of this review as well as the comparability between the studies included, the age range was set to include the period of early childhood and the temperament measures related to it. The study types of interest were observational studies either longitudinal or cross-sectional by design.

The following inclusion criteria were established:

1. Studies measuring the composition and/or diversity of the gut microbiome using either 16S rRNA or shotgun metagenomic sequencing techniques.

2. Studies that used well standardized questionnaires or laboratory assessments to measure temperament traits.
3. Studies focusing on healthy children under the age of 7 years.
4. Studies involving children born either via vaginal delivery or cesarean section.

The following exclusion criteria were established:

1. Studies written in any other language than English.
2. Meta-analyses, reviews, chapters or sections of books and studies that are not published in peer-reviewed journals.
3. Studies that did not measure temperament.
4. Studies that did not measure GM composition or did not use either 16S rRNA or shotgun metagenomic sequencing methods to assess the GM.
5. Studies including children with diagnosed gastrointestinal health conditions, genetic disorders, developmental disorder, learning disability or any acquired disabilities that are known to be associated with an altered GM composition and/or behavior.

2.3 Study selection and data extraction

Mendeley Reference Manager (v2.84.0) was used to collate articles from the PubMed database. One reviewer (MK) screened each article by title and abstract for eligibility according to the established inclusion and exclusion criteria. Articles that either met the exclusion criteria or did not meet the inclusion criteria were removed. The remaining full articles were then assessed for eligibility by MK. Ineligible articles were again excluded, resulting in the final set of articles chosen to be included in this review.

Preceding data extraction, a set of data variables of interest was established by MK and AA. This included details on author and journal, publication year, study design, participant demographics including sex and age, GM-associated techniques including collection method, the use of a buffer, sequencing method, hypervariable regions, exposure to antibiotics, method of temperament assessment, GM diversity and/or composition measures and results of associations between the different GM measures/microbial taxa and temperament as well as

other notable findings. The predefined data was then extracted into an Excel sheet for evaluation and synthesis.

3 RESULTS

Initial search of the PubMed database conducted in October 2022 yielded 24 articles. The second search conducted in February 2023 returned 25 articles with publication years ranging from 1997-2022. In the first phase, 11 articles were excluded for either not meeting all of the inclusion criteria or meeting one or more of the exclusion criteria based on abstract and title. Eight of these were review articles without original data. Only one of these review articles (Alving-Jessep et al., 2022) investigated associations between GM and early life temperament. One original study investigated associations between the GM and temperament in adults and as such did not meet all of the inclusion criteria. One study did not measure GM composition and one study did not measure temperament.

Full text screening was performed on 14 articles to determine eligibility. A total of seven studies did not measure either temperament or microbiota. Two of these were articles on studies that were planning on carrying out microbiome sequencing and temperament measurements in the future but had not published results yet. Three were commentaries on previous research and as such did not include original data. One article did not measure temperament and one did not measure GM composition. In the end, seven articles met the eligibility criteria, and were included in this systematic review (FIGURE 1).

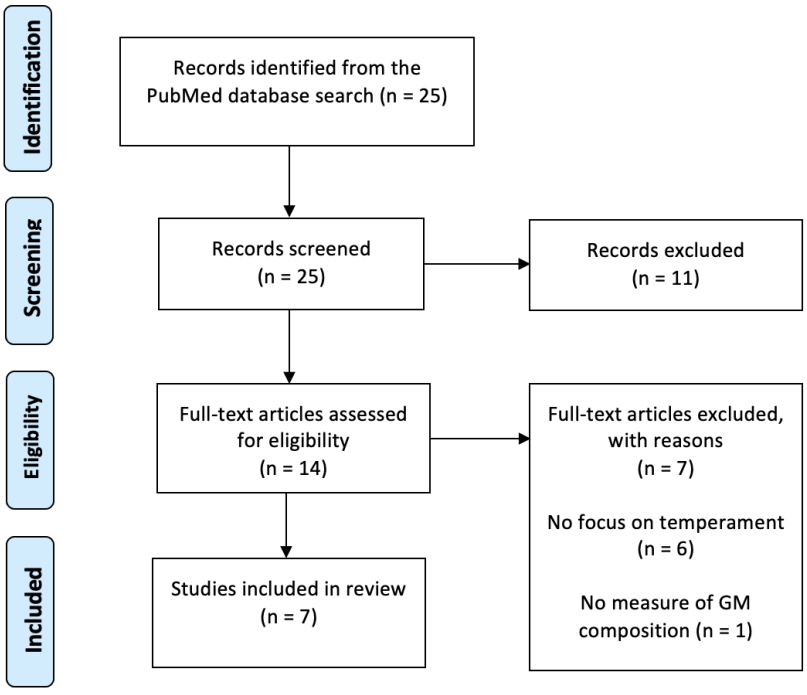

Figure 1 PRISMA flowchart describing the systematic review screening process

3.1 Study characteristics

The seven studies included in this review spanned a total of 563 unique participants. Two sets of two studies shared the same initial set of participants (Christian et al. 2015 & Delgadillo et al. 2022; Wang et al. 2020 & Xie et al. 2022). Wang et al. assessed the subjects (N=51) at a single timepoint at the age of one year. Xie et al. followed up on a subset of the participants (N=37) for a total of two years as well as including toddlers aged 2 years old (N=4) who had not completed a fecal sample at the age of 1 year. The number of participants in individual studies ranged from 51 to 301. Age of participants ranged from 9 days to approximately 29 months at time of assessment. Publication dates of the articles ranged from 2015 to 2022. Of all the studies, five were cross sectional observational studies and two were longitudinal in design (Fox et al., 2021; Xie et al., 2022). In both of the longitudinal studies, families were recruited during pregnancy and time of follow ups varied from the age of 1 week to 12 months (Fox et al., 2021) and from approximately the age of 1 year until 2 years of age (Xie et al., 2022). Four studies were conducted in the United States, two in China and one in Finland. Subjects were thus included from three continents (North America, Asia, Europe).

Table 1 Study settings

Authors	Country	Study type	Study population	N of boys	Age range (child)
Christian et al. 2015	United States	Cross-sectional observational	77 mother–toddler dyads.	41	18–27 months at time of assessment; mean age 23.14 (SD = 2.00).
Aatsinki et al. 2019	Finland	Cross-sectional observational	301 children (subcohort).	159	From birth followed until 6 months of age; mean age 65.2 days at the time of assessment.
Wang et al. 2020	China	Cross-sectional observational	51 mother-infant dyads.	20	Mean age of 12.3 (SD = 0.25) months.
Kelsey et al. 2021	United States	Cross-sectional observational	63 infants.	37	9 days to 56 days; mean age 25 days; median age 24 days.
Fox et al. 2021	United States	Longitudinal	Mother-child dyads. 67 infant donors, 91 samples collected at different ages (1–3 weeks, 2, 6, and 12 months).	35	From the age of 1 week followed until 12 months of age.
Delgadillo et al. 2022	United States	Cross-sectional observational	77 mother–toddler dyads.	41	18–27 months at time of assessment; mean age = 23.14 (SD = 2.00), with 91% falling between 21 and 26 months.
Xie et al. 2022	China	Longitudinal	51 infants at 1 year old and 41 toddlers at 2 years old were analyzed (of which a total of 37 toddlers completed fecal samples and temperament data both at the age of 1 year and 2 years).	20 at 1 year old. 16 at 2 years old. 12 boys completed a fecal sample both at 1 year old and 2 years old.	Mean age 12.3 (±0.25) months at the first time point and 27.5 ±1.43 months at the second time point.

3.2 Microbiome analyses

All studies included in this review analyzed the composition of the gut microbiota. In addition, Kelsey et al. explored the functional composition of the GM. Six out of the seven studies used 16s rRNA sequencing to analyze the gut microbiota and one study (Kelsey et al.) used shotgun metagenomic sequencing. Four out of the seven studies included used the Illumina MiSeq-platform for sequencing (Wang et al. and Xie et al. used the same data), two studies used the Roche 454 FLX Titanium System (Christian et al. and Delgadillo et al. used the same data) and one study employed shotgun metagenomic sequencing to analyze the microbiome with Illumina NovaSeq/HiSeq. Studies analyzing the 16s rRNA varied in their selection of hypervariable regions. Aatsinki et al. used V4; Fox et al., Wang et al. and Xie et al. used V3-4 while Christian et al. and Delgadillo et al. used V1-3 hypervariable regions. For identification of bacteria from the assigned Operational Taxonomic Units (OTUs), both the

GreenGenes database (Aatsinki et al., Christian et al., Delgadillo et al.) and Silva database (Fox et al., Wang et al., Xie et al.) were used. Kelsey et al. used a series of pipelines and functions developed in-house to analyze the microbiome. A pipeline, JAMSalpha, was used to acquire both taxonomic and functional relative abundances within each sample. To apply taxonomic classification, Kraken2 and k-mer analyses were used. Alpha diversity was measured in all studies except Wang et al while differences in overall community composition was measured in all studies.

Additionally, there was variety in sample collection methods. In three studies, the samples were first refrigerated at home at approximately 4°C for a maximum of 24 hours (Aatsinki et al., 2019; Christian et al., 2015; Delgadillo et al., 2022). Two studies transported the samples in a cooler (Fox et al., 2021; Wang et al., 2020), three studies kept the samples in ice (Christian et al., 2015; Delgadillo et al., 2022; Xie et al., 2022) and two studies did not specify the temperature at which the samples were transported (Aatsinki et al., 2019; Kelsey et al., 2021). After transportation, all but one study stored the samples at -80°C until sequencing; Aatsinki et al. extracted the DNA from the samples as soon as they arrived in the laboratory. Two studies used a stabilizing solution before freezing the samples; upon arrival in the laboratory Kelsey et al. immediately aliquoted the stool samples into cryovials containing a 20% Glycerol and 80% Phosphate-Buffered Saline solution; Fox et al. collected the stool samples at the ages of 2, 6, and 12 months at a laboratory site, where stool was transferred into OMNIgene gut collection kits (OMR-200, DNA Genotek) and the mixture aliquoted into cryovials before freezing.

Table 2 GM assessment methods

Authors	Microbiome sequencing technique	Hyper-variable region	Fecal sample collection method	Age of child at time of microbiome measurement
Christian et al. 2015	16S rRNA sequencing performed on the Roche 454 FLX Titanium System. PyNAST was used for sequence alignment with the GreenGenes core reference database.	V1-3	Samples were stored at home at approximately 4°C for up to 24 h after which they were transported in ice and stored at -80°C until pyrosequencing was conducted.	18-27 months
Aatsinki et al. 2019	16S rRNA sequencing conducted on the Illumina MiSeq platform. Downstream analysis with Qiime v1.9 pipeline, UCLUST was used to check sequences against the GreenGenes database and annotate OTU:s.	V4	Samples were stored at home in 4°C for up to 24 h after which they were transported in an unspecified temperature and sequenced.	2.5 months
Wang et al. 2020	16S rRNA sequencing using MiSeq platform. Sequences were clustered into OTUs with UPARSE. Taxonomy of each gene sequence was analyzed by the RDP Classifier algorithm against the SILVA database.	V3-4	After collection, samples were transported in a cooler (+4◦C, 1.5 h on average) to the laboratory and were frozen at −80◦C prior to further analysis.	12 months
Kelsey et al. 2021	Shotgun sequencing was analyzed using a series of pipelines and functions in the R language developed in-house and publicly available on Github under the package name JAMS.	NA (shotgun meta-genome)	The average time between infant reported defecation and freezing the samples was 7.96 h. Temperature unspecified during transport. Once received by the investigators, stool samples were immediately aliquoted into cryovials containing a 20% Glycerol and 80% Phosphate-Buffered Saline solution and stored at -80 ◦C.	9 to 56 days
Fox et al. 2021	16S rRNA sequencing conducted on Illumina MiSeq v3. DADA2 was used to cluster sequences into exact amplicon sequence variants and taxonomy was assigned based on the SILVA database.	V3-4	Stool was with sealed with film during transport. For home visits, the entire diaper was then sealed in a plastic bag and transported in cooler to the laboratory (max. 45 min). Visits at age 2, 6, and 12 months occurred at a clinical site with a laboratory where stool was trasferred into OMNIgene gut collection kits (OMR-200, DNA Genotek), the mixture aliquoted into cryovials, and stored at −80°C.	Stool was collected at a home visit 1–3 weeks after birth and at a clinical research site when the child was aged 2, 6, and 12 months
Delgadillo et al. 2022	16S rRNA sequencing performed on the Roche 454 FLX Titanium System. Downstream analysis with QIIME v1.8.0. PyNAST was used for sequence alignment and GreenGenes was used for taxonomic assignment.	V1-3	Samples were stored at home at approximately 4°C for up to 24 h after which they were transported in ice and stored at -80°C until pyrosequencing was conducted.	18-27 months
Xie et al. 2022	16S rRNA sequencing using MiSeq platform. Sequences were clustered into OTUs with UPARSE. Taxonomy of each gene sequence was analyzed by the RDP Classifier algorithm against the SILVA database.	V3-4	Fecal samples were collected with temperament data at home in accordance with the Human Microbiome Project (HMP) protocol. Samples were wrapped in ice packs and transferred to the refrigerator at -80◦C within 2 h.	1 year and 2 years

3.3 Temperament measures

Temperament was measured using well-established scales and questionnaires. All temperament measures used by the studies in this review were based on Rothbart's temperament framework. The choice of questionnaires varied according to the age group of the study participants. Infant Behavior Questionnaire -Revised (IBQ-R, see Putnam et al., 2014) was used in three studies (Fox et al., 2021; Wang et al., 2020; Xie et al., 2022) and its short form (IBQ-R SF) in two studies (Aatsinki et al., 2019; Kelsey et al., 2021). Two studies (Christian et al., 2015; Xie et al., 2022) used the Early Childhood Behavior Questionnaire (ECBQ, see Putnam et al., 2006). Delgadillo et al. investigated only the main temperament dimension of Effortful Control (EC), including five temperament subscales, and assessed by the Early Childhood Behavior Questionnaire short form (ECBQ-S) (Delgadillo et al., 2022).

Table 3 Temperament assessment methods

Authors	**Temperament measure**	**Age of child at time of temperament assesment**
Christian et al. 2015	Early Childhood Behavior Questionnaire (ECBQ) completed by the mother	18-27 months
Aatsinki et al. 2019	Infant Behavior Questionnaire - Revised short form (IBQ-R-SF), maternal report	6 months
Wang et al. 2020	Infant Behavior Questionnaire - Revised (IBQ-R), primary caregivers' report	12 months
Kelsey et al. 2021	Infant Behavior Questionnaire Revised Short Form (IBQ-R-SF), parental report	9 to 56 days
Fox et al. 2021	Infant Behavior Questionnaire - Revised (IBQ-R), maternal report	12 months
Delgadillo et al. 2022	The main temperament dimension of Effortful Control (EC) was measured with a composite of five temperament subscales assessed by the short form of the Early Childhood Behavior Questionnaire (ECBQ-S)	18-27 months
Xie et al. 2022	Infant Behavior Questionnaire-revised (IBQ-R) at 1 year of age. Early Childhood Behavior Questionnaire (ECBQ) at 2 years of age	1 year and 2 years

3.4 Results of GM and temperament analyses

3.4.1 Child temperament and GM alpha diversity

Alpha diversity, or the diversity of microbes within a given ecosystem, i.e., the gut of a single human subject, was measured in six of the seven studies included. A variety of different measures and indices were used to investigate species richness, i.e., number of different species, and species diversity i.e., differences in abundance between species; most common were the Shannon diversity index (SDI) used in six studies and the Chao1 richness index used in three studies. Other measures included the Simpson diversity index and a Faith's phylogenetic diversity measure (PG_Whole_tree) which were used by one study each.

Only two studies reported significant associations between alpha diversity and temperament measures, however, the results were disconcordant regarding temperament dimension (Table 4). Wang et al. did not report results concerning alpha diversity.

Christian et al. used the SDI and Faith's phylogenetic diversity measures to investigate alpha diversity separately for boys and girls at the age of 18-27 months. Age was not found to associate with any temperament variable; however, a significant association was found between both the SDI and phylogenetic diversity in boys but not in girls. Adjusting for age, a significant association was found between the composite scale of Surgency/Extraversion and phylogenetic diversity but not the between Surgency/Extraversion and SDI in both sexes. Among boys, phylogenetic diversity was also associated with two subscales loading on the composite scale of Surgency/Extraversion; phylogenetic diversity was associated with greater Sociability and greater High-Intensity pleasure, although the association to High-Intensity pleasure was attenuated when controlling for age. Among girls, higher SDI scores were significantly associated with lower scores on the composite scale of Effortful Control.

Aatsinki et al. found that in infants aged 6 moths, greater species diversity measured by the SDI was associated with lower negative emotionality as well as lower fear reactivity in an analysis adjusted for gestational age, infant age, sex, mode of delivery, breastfeeding, and antibiotics intake. In the unadjusted analysis, no such association was found. Species richness measured by the Chao1 index was not associated with temperament traits in the adjusted and unadjusted analyses. No sex differences were found. Regarding the covariates, SDI was

associated with breastfeeding status, the mode of delivery and mother´s age. Richness (Chao1) was associated with breastfeeding status.

Regarding alpha diversity, Kelsey et al. observed no significant associations between either the SDI or Chao1 indices for taxa and behavioral temperament in infants aged 9-56 days. Accordingly, significant associations between temperament traits and Chao1 functional terms (diversity, virulence factors, resistome, and GeneOntology (GO) terms) were not found. On the other hand, regarding GM diversity, both increased SDI and increased Chao1 indices were associated with increased homologous-interhemispheric connectivity. Additionally, increased homologic-interhemispheric connectivity was associated with increased negative emotionality. A significant indirect effect was observed, suggesting that the relationship between taxa diversity and negative emotionality may be mediated by homologous-interhemispheric connectivity. Furthermore, a significant association between increased virulence factor diversity (measured using Chao1) and increased negative emotionality as well as decreased regulation/orienting was observed. This suggests an indirect effect mediated by the effect of virulence factors on homologous-interhemispheric connectivity. These associations remained significant after adjusting for covariates (antibiotics, breastfeeding, delivery method, gestational age, head circumference at birth, infant age, infant weight at birth and at study visit, income, and sex).

Table 4 Findings regarding microbial alpha diversity

Authors	**Alpha diversity measures**	**Shannon diversity index (SDI)**	**Simpson diversity index**	**Chao1 richness index**	**Phylogenetic diversity (PD_Whole_tree)**
Christian et al. 2015	Shannon Diversity Index and Phylogenetic Diversity	**Effortful Control/Regulation** ↓(♀); *Sociability* ⇡(♂)			**Surgency/ Extraversion** ↑; *Sociability* ⇡(♂)
Aatsinki et al. 2019	Shannon Diversity Index and Chao1 (richness)	**Negative Emotionality** ↓; *Fear Reactivity* ⇣		No significant associations	
Wang et al. 2020	None given				
Kelsey et al. 2021	Shannon Diversity Index and Chao1 (richness)	No significant associations		No significant associations	
Fox et al. 2021	Shannon Diversity Index and Chao1 (richness)	No significant associations		No significant associations	
Delgadillo et al. 2022	Shannon Diversity Index	Analyzed only in relation to Effortful Control: no significant association was found			
Xie et al. 2022	Shannon Diversity Index and Simpson Index	No correlation between temperament dimensions and diversity indices was found at the age of two years. No difference in microbial diversity between 1 and 2 year-old toddlers.	No correlation between temperament dimensions and diversity indices was found at the age of two years. No difference in microbial diversity between 1 and 2 year-old toddlers.		

↑/↓ Positively/negatively associated with a **composite scale of temperament (Bold)**

⇡/⇣ Positively/negatively associated with a *subscale of temperament (Italic)*

♂ Association observed only in boys

♀ Association observed only in girls

3.4.2 Child temperament and GM composition – associations with beta diversity

Beta diversity, or the variation of the microbiome composition (GMC) in one environment compared to another, i.e., difference in taxonomic abundancy profiles between different samples, was measured in all of the studies included in this review. Wang et al. did not report results of microbial beta diversity in relation to temperament. Methods used for comparing beta diversity included Permutational Analysis of Variance (PERMANOVA), Analysis of Similarities (ANOSIM) and clustering with the Partitioning Around Medoids (PAM) algorithm. Distance metrics used included the Bray-Curtis dissimilarity, weighted (accounts for abundances of taxonomic units) and unweighted (presence/absence of taxonomic units only) UniFrac, and Deicode (a form of Aitchison distance). Results were typically visualized using the Principal Coordinates Analysis (PCoA).

Three studies reported an association between beta diversity and Surgency or it's subscales such as High-Intensity Pleasure (Aatsinki et al., 2019; Christian et al., 2015; Fox et al., 2021). All of the studies used different distance or dissimilarity metrics and analysis methods.

Christian et al. measured beta diversity in children aged 18-27 months using weighted and unweighted UniFrac distance variables and PERMANOVA. They observed that the composite scale of Surgency/Extraversion was associated with a specific microbiota structure in boys when measured by the unweighted UniFrac distance metric. Loading on the association in boys were the subscales of Sociability, High-Intensity Pleasure, and Activity Level. Only the subscale of High-Intensity Pleasure, loading onto Surgency/Extraversion, was found to be significantly associated with a distinct microbial community structure when including OTU abundances via the weighted UniFrac metric.

Aatsinki et al. assessed the composition of the GM at the age of 2.5 months by clustering the subjects according to their GMC profiles using the Bray-Curtis distance metric based on OTU counts and the PAM clustering method. The Bifidobacterium/Enterobacteriaceae cluster exhibited the highest scores in the temperament composite scale of Regulation as well as in the subscales of High-Intensity Pleasure, Cuddliness and Duration of orienting. Conversely, the lowest scores in each of the previously mentioned temperament measures were observed in the Bacteroides cluster.

Fox et al. investigated beta diversity differences by age group using PERMANOVA and adjusting for subject. The results were visualized using PCoA plots of the microbial beta

diversity measured by the DEICODE distance metric. An association between gut microbial beta diversity and the composite scale of Surgency/Extraversion as well as its subscales Approach, High-Intensity Pleasure and Smiling/Laughter was found in infants aged 1-3 weeks.

Table 5 Statistically significant associations between microbial beta diversity and temperament

Authors	Beta diversity measures	Weighted UniFrac	Unweighted UniFrac	Deicode	Bray-Curtis dissimilarity
Christian et al. 2015	Weighted and Unweighted UniFrac distance (PERMANOVA, QIIME)	*High-Intensity Pleasure*	**Surgency/Extraversion;** *Sociability* (♂); *High-Intensity Pleasure* (♂); *Activity Level* (♂); *Fear* (♀)		
Aatsinki et al. 2019	Bray-Curtis dissimilarity (Clustering, Partitioning Around Medoids (PAM))				**Regulation**; *High-Intensity Pleasure*; *Cuddliness*; *Duration of Orienting*
Wang et al. 2020	Bray–Curtis distance matrix (Principal coordinate analysis (PCoA), PERMANOVA)				Results not reported in relation to temperament
Kelsey et al. 2021	Predicted proteomes of each metagenomic sample were acquired using InterproScan and BLASTp (VFDB and ResFinder databases) after which the relative abundance in parts per million (PPM) of each feature was used. No beta diversity results were reported.				
Fox et al. 2021	DEICODE distance (PERMANOVA)			**Surgency/ Extraversion** (age 1-3 weeks); *Approach* (age 1-3 weeks); *High-Intensity Pleasure* (age 1-3 weeks); *Smiling/Laughter* (age 1-3 weeks); *Sadness* (age 12 months)	
Delgadillo et al. 2022	Bray-Curtis distance (PERMANOVA, ANOSIM)				Analyzed only in relation to Effortful Control: No significant differences in community structure between high and low EC groups on the phylum level or on the genus level were found
Xie et al. 2022	Unweighted UniFrac distance		No significant associations found		

Composite scales of temperament (Bold)

Subscales of temperament (Italic)

3.4.3 Child temperament and GM composition – taxonomic level associations

Taxa-level analyses of the associations between gut microbes and temperament were included in all of the studies involved in this review. Both genus and species level associations were reported as well as associations on the operational taxonomic unit level.

There was large heterogeneity in the taxa associations. However, three studies reported a positive association between genera belonging to Bacillota (Firmicutes) and Surgency/Extraversion or its subscales and two studies found an association with lower Negative Affectivity. However, five different genera belonging to Bacillota were conversely associated with either lower Surgency/Extraversion or higher Negative Emotionality or its subscales. Likewise, three studies reported positive associations between two taxa in Actinomycetota and Positive Emotionality, however two genera belonging to this phylum were found to associate with higher Negative Affectivity. Three taxa in the Pseudomonatota phylum were found to associate with higher Negative Emotionality. There were no other apparent concordant results on the phylum-level.

On the genus level, *Bifidobacterium* was positively associated with higher Surgency/Extraversion in three studies and negatively associated to Negative Affectivity in one study. One study found two species of the genus *Bifidobacterium* to associate with higher Effortful Control and one genus was also observed to associate with higher Negative Affectivity. Additionally, an undefined genus in the *Lachnospiraceae* family was positively associated with Surgency/Extraversion in one study as well as three different genera belonging to *Lachnospiraceae* being positively associated with subscales loading onto Surgency/Extraversion and Effortful Control in another study. Lachnospiraceae was not associated with higher Negative Affectivity. *Dialister* was found by one study to associate with higher Effortful Control as well as a subscale of Surgency/Extraversion in another study. Findings regarding *Ruminococcaceae* were mixed, with two studies observing an association either with lower Surgency/Extraversion or higher Negative Emotionality and one study observing an association with higher Sociability in boys.

Table 6 Statistically significant associations between bacterial genera/species and composite scales of temperament after adjusting for covariables

Taxonomy			Temperament scale		
Phylum	**Family**	**Genus (and Species)**	**Surgency/ Extraversion**	**Negative Affectivity**	**Effortful Control/ Regulation**
Actinomycetota	*Bifidobacteriaceae*	*Bifidobacterium*	↑**[2]; ↑[3]; ↑[5]	⇣[7]	
		Bifidobacterium catenulatum			↑[4]
		Bifidobacterium pseudocatenulatum		↑[4]	↑[4]
	Coriobacteriaceae	*Atopobium*	↓**[2]	↑**[2]	
		Collinsella	↑[5]		
	Micrococcaceae	*Rothia*		↑**[2]	
Aquificota	*Desulfurobacteriaceae*	*Thermovibrio guaymasensis*		↓[4]	
Bacillota (Firmicutes)	*Acidaminococcaceae*	*Acidaminococcus*		↑[5]	
	Clostridiaceae	*Hungatella*		↓[3]	
	Lachnospiraceae	*Anaerostipes*	⇡[7]		
		Undefined genus	↑[5]; ⇡[7]		⇡[7]
	Lactobacillaceae	*Lactobacillus*		↓[5]	
	Peptoniphilaceae	*Peptoniphilus*		⇡**[2]	
	Selenomonadaceae	*Megamonas*		↑[5]	
	Ruminococcaceae	*Faecalibacterium*	↓[7]		
		Ruminococcus-1		↑[5]	
		Undefined genus	⇡♂*[1]		
	Streptococcaceae	*Streptococcus*	↑[2]		
	Veillonellaceae	*Dialister*	⇡♂[1]		↑[6]
Bacteroidota	*Rikenellaceae*	*Alistipes*			↓[6]
		Undefined genus	⇡♂[1]	⇡♀[1]	
Pseudomonatota	*Enterobacteriaceae*	*Klebsiella*	↓[5]	↑[5]	
	Erwiniaceae	*Erwinia*		↑**[2]	↑[2]
	Tannerellaceae	*Parabacteroides*	⇡♂[1]		
	Yersiniaceae	*Serratia*		↑**[2]	
Verrucomicrobiota	*Akkermansiaceae*	*Akkermansia*			↑[6]

[1] Christian et al. 2015; [2] Aatsinki et al. 2019; [3] Wang et al. 2020; [4] Kelsey et al. 2021; [5] Fox et al. 2021; [6] Delgadillo et al. 2022; [7] Xie et al. 2022

↑/↓ Positively/negatively associated with the composite scale

⇡/⇣ Positively/negatively associated with one or more subdimensions loading on the composite scale

♂ Association observed only in boys

♀ Association observed only in girls

* Attenuated when adjusted for age

** Attenuated when adjusted for gestational age, sex, mode of delivery, infant age, breastfeeding and antibiotics intake

4 ASSOCIATIONS BETWEEN ANTIBIOTICS EXPOSURE, GM DIVERSITY/COMPOSITION MEASURES, AND TEMPERAMENT

Information on antibiotic exposure was collected in four of the seven studies. All of the studies did not, however, explicitly report associations between antibiotics, GM diversity/composition, and temperament measures. No direct associations between antibiotic exposure and GM diversity were observed. Furthermore, no association between GM beta diversity and antibiotics exposure was found. Kelsey et al. did, however, find a statistically significant association between resistome diversity, as measured by the Chao1 index for resistome, and antibiotics administered at the hospital after birth in infants aged 9-56 days. They were also the only study to explore metagenomics.

Table 7 Associations between antibiotics, GM Diversity/Composition, and Temperament

Authors	**Antibiotic treatments**	**Antibiotic treatments and GM diversity/ composition**	**Antibiotic treatments and temperament**
Christian et al. 2015	Excluded	Excluded	Excluded
Aatsinki et al. 2019	Mothers provided information on infant antibiotic treatments. 37 infants (12.3%) had been treated with an antibiotic by the time of stool sample collection. Antibiotic exposure was included as a covariate in statistical analyses.	Not reported	No significant associations
Wang et al. 2020	Excluded	Excluded	Excluded
Kelsey et al. 2021	30 infants (48%) had received antibiotics by the time of sequencing. This included infants potentially exposed to antibiotics during the prenatal period (12%), labor and delivery (41%) and through administration of antibiotics directly to the infant between birth and study appointment (only 4 infants or 6%). Antibiotic exposure was included as a covariate in statistical analyses.	Infants aged 9-56 days: resistome diversity (Chao1 index for resistome) was significantly associated with antibiotics administered at the hospital after birth	Not reported
Fox et al. 2021	26 children (39%) had received an antibiotic or antifungal by the time of sequencing. Statistical analyses were not adjusted for antibiotic use, as it had no significant statistical relationship with microbiome composition at any timepoint.	Antibiotics use had no significant association with microbial beta diversity in any subgroup. Consequently multivariate models were not adjusted for antibiotics use.	Not reported
Delgadillo et al. 2022	Excluded	Excluded	Excluded
Xie et al. 2022	Infant antibiotics exposure and probiotics consumption during 6–12 months and 13–24 months were reported by mothers. 28 infants (54.90%) and 31 toddlers (75.61%) had received antibiotics by the age of 1 and 2 years, respectively. Antibiotic exposure was included as a covariate in statistical analyses.	At 2 years old, no significant association between antibiotic exposure and GM alpha or beta diversity were observed.	Scores of having antibiotics were higher than non-antibiotics on fear and frustration at 2 years old but not at 1 year old.

5 DISCUSSION

The main objective of this review was to gather and synthetize evidence regarding possible associations between GM diversity, composition, and childhood temperament traits in current research. Some interesting patterns were found to emerge. Surgency/Extraversion was observed to associate with a distinct gut microbial composition in three different studies. On the taxa-level, an interesting pattern of association between three known butyrate-producing bacteria belonging to the phylum Bacillota (Firmicutes) and temperamental traits was observed. Additionally, significant heterogeneity was observed regarding study design and methodology.

5.1 Findings regarding GM diversity

Only two studies found statistically significant associations between microbiota alpha diversity measures and temperament scales. Christian et al. observed an association between greater phylogenetic diversity and higher Surgency/Extraversion while Aatsinki et al. found greater diversity to associate with lower Negative Emotionality and lower Fear Reactivity in their adjusted models. Aatsinki et al. studied infants under 6 months of age while the mean age for toddlers assessed by Christian et al. was close to 2 years. Lower diversity of the gut microbiota is typically associated with active breastfeeding and tends to increase with age as the child's nutrition becomes more heterogenous (Bäckhed et al., 2015). This could partially explain why the associations between diversity and temperament were only noted by Aatsinki et al. in models adjusted for covariables including breastfeeding. These associations were, however, not replicated in later studies either in children over or under the age of 6 months, which is the age recommended by the WHO for beginning of complementary feeding.

Kelsey et al. were, as noted before, the only study to explore metagenomics as well as functional brain network connectivity. Interestingly, they found significant indirect associations between taxa-level alpha diversity and temperament measures mediated by the measured activity of specific brain functional networks. Contrary to the findings of Christian et al. and Aatsinki et al., higher alpha diversity (Shannon diversity and Chao1 richness) was found to be associated with increased Negative Emotionality when mediated by the homologous-interhemispheric network. This association did not, however, remain significant after adjusting for covariables. Additionally, gut microbiome functional diversity (virulence factor diversity) was associated with both higher Negative Emotionality and lower Regulation/Orienting when mediated by measured activity of the homologous–

interhemispheric neural network. Virulence factor diversity was also positively associated with taxa diversity. Regarding the contradictory findings with Christian et al. and Aatsinki et al., one possible explanation presented by Kelsey et al. is that certain components of behavioral temperament, such as fear behaviors, do not present similarly in the youngest of infants. The mean age of assessment for Kelsey et al. was 25 days, earliest in the studies reviewed. As noted by Alving-Jessep et al. in their review, the pattern of the relationship observed by Kelsey et al. could suggest that increased homologous-interhemispheric connectivity at the early stage of development is an aberrant response, not to be expected later in childhood (Alving-Jessep et al., 2022). The effect of alpha diversity on temperament may be different as the brain develops. Related, certain features of behavioral temperament, such as fear behaviors, are observed to emerge only later during the first year of life (Grossmann & Jessen, 2017). Furthermore, greater taxa-level alpha diversity was found by Kelsey et al. to associate with increased fronto-parietal connectivity. Previous infant studies have observed a similar positive association between taxa diversity and parietal cortex structure and function (Carlson et al., 2018; Gao et al., 2019). Although no significant indirect association between fronto-parietal connectivity and temperament measures was reported in the study by Kelsey et al., the fronto-parietal network has previously been associated with increased Regulation and Orienting in infants as well as decreased internalizing disorders in adulthood (Kaiser et al., 2015). In a recent study, alpha diversity was found to be negatively associated with depressive symptoms in two different cohorts (Radjabzadeh et al., 2022). The possibility presented by Kelsey et al., that the effect of gut microbiota on brain network connectivity may precede direct associations with behavioral traits is interesting but requires further research to validate or exclude the hypothesis.

In summary, the amount of evidence supporting a link between GM alpha diversity and temperament remains limited as also noted by Alving-Jessep et al. in their systematic review (Alving-Jessep et al., 2022). The tentative associations observed by Christian et al. and Aatsinki et al. were not replicated in later studies included in this review. There was, however, significant heterogeneity in study populations, designs, measures, and covariates included in the analyses between different studies which may contribute to the mixed findings. Furthermore, the total amount of research on the subject remains limited as well. As such, possible links between GM diversity and temperament traits in early childhood remain inconclusive, and further studies are needed to exclude or verify the previously observed associations or lack thereof. To establish possible causality, longitudinal studies would be

required. Metagenomics further expands the scope of research allowing observations on the functional diversity of the microbiota to be made. This dimension of GM diversity cannot be assessed in studies employing 16s RNA techniques. The number of studies regarding associations between early life GM and temperament incorporating metagenomics is currently limited. More research on this important aspect of the GM is needed. Furthermore, the mechanisms by which specific microbial taxa may impact behavior remain unclear and further research on this is required to understand the effects of microbial functional properties and diversity on host biomechanisms and behavior. Finally, the indirect associations observed by Kelsey et al. present an interesting idea of a possible link that remains to be further explored.

5.2 Findings regarding GM composition

5.2.1 GM beta diversity and temperament

Results linking temperament traits to distinct microbial community structures were limited, and similar findings were only made in relation to the composite scale of Surgency/Extraversion. Christian et al. observed a sex-dependent association with higher Surgency/Extraversion and a specific microbiota structure in boys aged approx. 2 years when measured by the unweighted UniFrac distance metric. Aatsinki et al. found a distinct microbial structure dominated by *Bifidobacterium* and *Enterobacteriaceae* to associate with several of the subscales loading onto Surgency/Extraversion in infants approximately 2 months of age, when using clustering and measured by the Bray-Curtis distance metric. Fox et al. found a similar association between GM beta diversity and Surgency/Extraversion in infants aged 1-3 weeks as measured by the Deicode distance metric. Summarizing, these findings can be seen as relatively consistent when considering that some of the studies did not report findings regarding beta-diversity. As beta-diversity level findings were mostly reported without further analysis of the associated unique microbial structures, it is hard to make hypotheses of the possible mechanisms behind these associations.

5.2.2 Taxonomic level findings

Most of the associations between individual bacterial genera and temperament traits were only observed in one individual study. Several possible factors may influence this finding,

including i.e., cultural differences in nutrition, environment (rural vs urban), antibiotic prescriptions and methodological choices. Age is also a significant factor impacting GM composition. The gut microbiota goes through relatively rapid changes during the first years of life. Soon after birth, the GM is typically characterized at the family level by relatively high levels of *Enterobacteriaceae, Bifidobacteriaceae*, and *Clostridiaceae*, but low levels of *Lachnospiraceae* and *Ruminococcaceae*. By around the age of 1-3 years, coinciding with weaning and the shift to solid foods, strict anaerobes have been found to take over as the dominant taxa, and GM diversity increases to adult-like levels (Koenig et al., 2011; Palmer et al., 2007; Yatsunenko et al., 2012). The age of study subjects in this review ranged from 24 days to over 2 years at time of assessment, which may in part contribute to the heterogeneity of results on the taxa-level.

Four different bacterial genera were found to have significant associations with temperament traits by more than one study. Only the families *Bifidobactericeae*, *Lachnospiraceae*, *Ruminococcaceae* and genus *Dialister* showed consistency in their observed temperament associations between studies. Most of the concordant findings were made in relation to higher Surgency/Extraversion, but in the case of *Bifidobactericeae* also to higher Effortful Control/Regulation. Surgency is known to predict both more extraversion and higher self-regulation in toddlerhood (Komsi et al., 2006). Conversely, lower Positive Emotionality in early childhood has been observed to predict depressotypic cognitive styles in middle childhood (Hayden et al., 2006). *Bifidobacteriaceae* have been found to typically present in relative abundance soon after birth in addition to *Enterobactericeae* and *Clostridiaceae*, while *Lachnospiraceae*, on the other hand, is typically observed only in low levels in infants with abundances generally increasing with age (Bokulich et al., 2016; Chu et al., 2017; Yassour et al., 2016).

Both Wang et al. and Fox et al. found the genus *Bifidobacterium* to be positively associated with the composite scale of Surgency/Extraversion as well as some of its subscales during the first year of life. Aatsinki et al. found a similar association, although this did not remain statistically significant after controlling for all covariables. All of the significant associations of *Bifidobacteriaceae* with Surgency/Extraversion were observed in infants with a mean age of under or approximately 1 year at the time of assessment. Additionally, at the cluster level Aatsinki et al. found a cluster dominated by *Bifidobacterium* and *Enterobactericeae* to present with the highest scores in the composite scale of Regulation. In toddlers aged 2 years, Xie et al. found *Bifidobacterium* to associate negatively with Perceptual Sensitivity, a subscale

loading onto the composite scale of Negative Affectivity. Contrary to the other studies, Kelsey et al. found an abundance of the genus *Bifidobacterium pseudocatenulatum* to associate with higher Negative Emotionality. The subjects of this study were the youngest with a mean age of 25 days. Consistent with the findings of Aatsinki et al., the genera *Bifidobacterium pseudocatenulatum* as well as *Bifidobacterium catenulatum* were also positively associated with Regulation/Orienting.

Bifidobacteriaceae is a family of essential bacteria responsible for utilizing human milk oligosaccharides otherwise indigestible (Gnoth et al., 2000). In infants, human milk oligosaccharides promote the growth of beneficial bacteria in the gut while at the same time preventing colonization of pathogenic bacteria (Lievin et al., 2000). Thus, *Bifidobactericeae* is thought to play an important role in fighting infections. The results found in the studies are relatively consistent with each other and thus suggest that *Bifidobactericeae* could be a potential predictor of infant temperament. Considering the observed relationship between Surgency/Extraversion in infancy and Surgency in later childhood, these results point to a potential significance of this genus of bacteria in the development of higher pro-social behavior/extraversion as well as emotional regulation in later childhood.

In studies examining the potential effect of GM composition and probiotic supplementation on diagnosed psychiatric disorders in children and adolescents, infant supplementation of prebiotics and micronutrients has been shown to alter the number of *Bifidobacterium* in the gut. One study observed that children affected with Attention Deficit and Hyperactivity Disorder (ADHD) or ASD had a significantly lower number of *Bifidobacterium* species in feces during the first six months of life. Moreover, a significant reduction in the occurrence of ADHD or autism spectrum disorder (ASD) was observed in children whose mothers received a probiotic treatment (Lactobacillus Rhamnosus GG) 4 weeks before labour as well as the children themselves during the first 6 months versus control group. (Pärtty et al., 2015). Another study observed a consistent increase over time in the prosocial behavior of children diagnosed with ASD when a combination of a galactooligosaccharide treatment (B-GOS) and an exclusion diet was used (Grimaldi et al., 2018). Bifidobacterial populations have also been found to increase following administration of micronutrients in individuals with ADHD (Stevens et al., 2019).

The bacterial family of *Lachnospiraceae* was found by Fox et al. to positively associate with the composite scale of Surgency/Extraversion as well as several of the subscales loading onto

it in infants. Xie et al. found a similar positive association between unspecified *Lachnospiraceae* and two of the subscales loading onto the composite scale of Surgency as well as Anaerostipes (a genus belonging to *Lachnospiraceae*) and Surgency in toddlers aged 2 years. In addition, an association between undefined *Lachnospiraceae* and a subscale of Effortful Control/Regulation was also observed by Xie et al.

Lachnospiraceae, a family of bacteria belonging to the phylum Bacillota (Firmicutes), is one of the key butyrate producing taxa in the GM (S. Liu et al., 2019). Butyrate is a short-chain fatty acid (SCFA) believed to play a vital role in maintaining gut homeostasis and epithelial integrity, since it is the primary energy source of colonocytes, interferes with proinflammatory signals, and directly influences host gene expression by inhibiting histone deacetylase activity (Cremin et al., 2003; Hamer et al., 2008). Butyrate has also been found to regulate colonic motility and increase colonic blood flow (Velázquez et al., 1997). In animal models, butyrate-producing bacteria have been shown to improve cognitive performance, social behavior, and mental health (Stilling et al., 2014).

In previous literature, *Lachnospiraceae* have been found to present in decreased quantities in children diagnosed with ASD compared to neurotypical children (S. Liu et al., 2019; Ma et al., 2019). Furthermore, following administration of a prebiotic galactooligosaccharide treatment (B-GOS), a significant increase in the number of *Lachnospiraceae* was observed in conjunction with improvements in anti-social behaviour, and significant changes in fecal and urine metabolites in autistic children (Grimaldi et al., 2018). This is consistent with the known ability of some *Lachnospiraceae* strains to produce butyrate as a metabolite from non-digestible food ingredients. In a recent study of 121 adult patients with diagnosed depression, butyrate-producing bacteria (Coprococcus and Dialister) were found to be significantly depleted in the gut (Valles-Colomer et al., 2019). Another recent study examining links between the GM and depression in two large cohorts found, that of the 12 significantly associated genera 10 belonged to the families *Ruminococcaceae* and *Lachnospiraceae*. Most of the significantly associated genera in the Lachnospiraceae family had a positive association i.e., the number of bacteria belonging to these genera was increased in individuals with higher depressive symptoms, however the genus Lachnospiraceae UCG001 was found to have a negative association with depression (Radjabzadeh et al., 2022). In 2-year-old children, unspecified *Lachnospiraceae* was found to present in increased numbers in children with internalizing problems. On the OTU level, the group of *Lachnospiraceae* was mostly composed of two OTUs classified to the *Lachnospiraceae* NK4A136 group in this study

(Loughman et al., 2020). Another study also found the presence of unidentified *Lachnospiraceae* to positively correlate with ASD severity in children with diagnosed ASD (Ding et al., 2020). As previously noted, levels of *Lachnospiraceae* are generally low in younger children and increase with age.

Dialister, another member of the phylum Bacillota (Firmicutes), was found by two studies to associate with temperament traits. Delgadillo et al. observed an association between *Dialister* and higher Effortful Control/Regulation. It needs to be noted, however, that Delgadillo et al. investigated only associations to this dimension of temperament. Christian et al. found *Dialister* to associate with higher Activity Level and increased High-Intensity pleasure, both subscales of Surgency/Extraversion. *Dialister* is another butyrate producer and has been found to be depleted in the GM of individuals with depression (Valles-Colomer et al., 2019).

Finally, *Ruminococcaceae*, also of the phylum Bacillota and a known butyrate producing bacteria, was found to associate with temperament traits by more than one study. Christian et al. found *Ruminococcaceae* to associate with Sociability, a subscale of Surgency/Extraversion, although this association was attenuated by the inclusion of age in the statistical model. Conversely, Fox et al. found *Ruminococcaceae* to associate with higher Negative Emotionality. Several studies have reported lower levels of *Ruminococcus* in children diagnosed with ASD compared to controls (De Angelis et al., 2013; S. Liu et al., 2019; Niu et al., 2019). In a recent study, the family *Ruminococcaceae* was found to significantly associate with depressive symptoms in two different cohorts. All significantly associated genera of *Ruminococcaceae* were found to be depleted in individuals with higher depressive symptoms. (Radjabzadeh et al., 2022). Previously, Ruminococacceae have been repeatedly found to be depleted in cases of both uni- and bipolar depression (Cheung et al., 2019; Hu et al., 2019; R. T. Liu et al., 2020; Simpson et al., 2021; Valles-Colomer et al., 2019). Strains of *Ruminococcacceae* have also been found to be depleted in individuals with inflammatory bowel disease (Nagao-Kitamoto & Kamada, 2017). Results found in this review regarding the possible impact of *Ruminococcaceae* on temperament are mixed and warrant further investigation. Of note is also, that the genus *L-Ruminococcus,* is assigned to the *Lachnospiraceae* family and not all genera of *Ruminococcaceae* were specified in the studies reviewed. Given the known role of *Ruminococcaceae* in producing butyrate and considering the many previously observed negative associations with depressive symptoms in adults, this family of bacteria presents a possible target for future research.

The findings presented in this review suggest that an abundance of *Bifidobacteriaceae* in infancy may promote Surgency/Extraversion in the first year of life as well as Effortful Control/Regulation into toddlerhood. Additionally, results implicate a possible role of *Lachnospiraceae* in increasing Surgency/Extraversion, supporting previously observed associations to neurological and psychiatric disorders in older children and adults. The association of three butyrate-producing genera of *Lachnospiraceae*, *Ruminococcaceae* and *Dialister* with temperament traits supports the role of this short-chain fatty acid in microbiota-gut-brain crosstalk. It is noteworthy, that Alving-Jessep et al. observed in their systematic review of early life GM composition/diversity and temperament a similar "…pattern of positive temperament traits being associated with GM communities biased toward short-chain fatty acid production from a metabolism based on dietary fiber and complex carbohydrates…" (Alving-Jessep et al., 2022). Both studies included papers absent from the other, and in both cases these papers included observations on butyrate-producing bacteria associating with temperamental traits. Previous studies on both humans and animals have shown alterations in the levels of SCFAs in many diseases, including neurological and psychiatric disorders such as autism spectrum disorder and major depressive disorder as well as neurodegenerative diseases such as Parkinson disease. SCFAs may be either directly or indirectly involved in communication along the MGB axis both through their neuroactive properties and their influence on other gut–brain signalling pathways including the immune and endocrine systems (Dalile et al., 2019). Considering the previously observed associations of *Lachnospiraceae*, *Ruminococcaceae* and *Dialister* with depressive symptoms in adults, the results found in this review may support an association with possible later life psychopathology but are, however, mixed and warrant further research. Regarding the family *Lachnospiraceae*, the partially mixed results in literature may owe to some of the genera in this family being butyrate-producing "fibre fermenters" while others are not (Loughman et al., 2020). Furthermore, some strains of *Lachnospiraceae* produce also other metabolites such as formate and lactate or H2 in addition to butyrate. However, especially the *Roseburia/Eubacterium rectale* group of *Lachnospiraceae* can produce butyrate without H2 especially at mildly acidic pH, along with the consumption of acetate. This requires cooperation with acetate-producing bacterial species (Vacca et al., 2020) one of which is *Bifidobacterium* (Ríos-Covián et al., 2016). The *Lachnospiraceae* family is a heterogeneous taxon both phylogenetically and morphologically, and the abundance of this family also been observed to increase in association to different diseases, even though taxa belonging to this family have repeatedly displayed their ability to produce metabolites beneficial for the host.

(Vacca et al., 2020). These observations highlight the complexity of the functional mechanisms of bacteria. Consequently, the functional mechanisms of bacteria behind observed effects on host behavior warrant further elucidation through metagenomic analyses. In order to establish individual genera of bacteria as behavioral biomarkers, further studies are needed to replicate the previously observed associations. Additionally, knowing that certain bacteria can have either harmful or beneficial effects depending on the presence or absence of other bacteria and environmental circumstances, bacterial interactions such ass cross-feeding should be better understood and integrated in the interpretation of results.

5.3 Findings regarding the effects of antibiotics on GM diversity/composition and temperament

Antibiotics exposure was not found to have a significant association with GM diversity or composition in any of the studies taking antibiotics into account. Antibiotics exposure was, however, found by Kelsey et al. to associate with increased resistome diversity i.e., the ability of bacteria to resist antibiotics. As the increase in resistome was only observed in a single study (other studies did not investigate metagenomics), no such conclusions can reliably be made. Furthermore, as this was a cross-sectional study, no conclusions about the persistence of this effect over time can be made.

5.4 Limitations of the included studies and the current review

Several limitations of the studies were identified, possibly explaining some of the variation of results. First, the diversity of covariables included in the statistical models of different studies complicates the analysis and comparability of results between different studies. The moderating effect on age on the GM is well known and likely explains some of the variation in GM diversity as well as in the taxa-level findings. Considering the well-known and relatively rapid changes in GM composition during the first years of life, the age-range between subjects in different studies was relatively large and most likely contributes to the differing findings regarding GM composition. Furthermore, various environmental influences can affect the microbiota of infants and children. Environmental factors with a potential to alter the microbiota in infants and children range from birth mode, diet and antibiotic use to stress and pet ownership (for a summary, see e.g., Cryan et al., 2019). Although the influence on microbiota composition of some of these factors diminishes over time, these early-life

factors may still have a significant impact on the mental and physical health of a person. This is based on the hypothesis that early life alterations of the GM may influence the developing brain during sensitive periods of microbiota-gut-brain interactions, thus possibly altering the trajectories of mental health and cognitive performance development. (Cryan et al., 2019). A more homogenous set of covariables would increase comparability between different studies, but currently no consensus of a standard or preferred set of covariables exists. The application of covariables should ideally be based on observed impacts of these variables on GM and/or temperament measures. In order to establish a preferred set of covariables, a systematic literature review summarizing observed associations between different environmental factors and the GM and/or temperament would be beneficial. Further discussion on this subject is needed.

For preventing shifts in microbial composition in stool samples, immediate freezing of samples to -80°C is considered the "gold standard". However, this approach might not be feasible for use in remote areas where cold chain transport is not reliable, or in studies that request subjects to send ambient-temperature household samples to laboratories. When cold chain transport is not feasible, proper preservation of stool samples is essential to minimize microbial community shifts and inactivate infectious agents. However, it is not well known how the usage of a preservation solution after collection of fecal samples can impact results of GM composition, and potentially creating biases in downstream analyses. (Chen et al., 2019). The studies in this review varied regarding time before freezing and temperature during transport, as well as the choice of using a preservation solution. This can potentially impact results of GM sequencing.

Furthermore, regarding GM sequencing, the choice of hypervariable region has been found to influence GM diversity and composition. In a study comparing reads acquired with the usage of three different hypervariable regions of the 16S rRNA gene (V1-2, V3-4, V4), the sensitivity of primer sets for identifying the overall microbial community and certain bacterial taxa differed from each other. Reads generated by the V4 primer pair, for example, showed a higher level of alpha diversity in the gut microbial community compared with other hypervariable regions and the degenerate 27f-YM primer failed to detect most of the *Bifidobacteriaceae*. (Chen et al., 2019). All of the previously mentioned hypervariable regions were represented in the studies examined, possibly creating a bias in the results. Another study also found that when using unsuitable primer combinations, outdated reference databases, or inadequate pipeline settings, some bacterial genera were found to be

underrepresented or even missing in taxonomic profiles. Concerning this, they concluded that quality thresholds as well as bioinformatic settings are responsible for several observed differences between studies using 16S rRNA sequencing and highlighted the importance of a thought-out study design with sufficiently complex mock standards and appropriate V-region choice for the sample of interest as well as testing the use of processing pipelines and parameters beforehand. (Abellan-Schneyder et al., 2021).

Additionally, an important limitation of studies employing 16s RNA sequencing techniques is the lack of consideration for the functional capacities and functional diversity of microbes. Bacteria vary in their capacity to influence host responses. These functional capacities are the mechanisms by which the bacteria influences function of the gut-microbiota-brain axis and an understanding of these mechanisms increases continuously. To explore the functional aspect of the GM, more studies incorporating metagenomic and metabolomic techniques are needed. Additionally, bacterial interactions such ass cross-feeding should be better understood and integrated in the interpretation of results.

In order to establish causality regarding the impact of the GM in developmental trajectories, a longitudinal approach would be highly beneficial. Ideally, this would entail taking measurements of both the GM and temperament concurrently at multiple time-points. The variation in the methodologies used in the studies presents a challenge to the comparability of results. Significant heterogeneity was found in the data collection, fecal sample processing, GM analysis pipelines and inclusion/exclusion of covariables in statistical analyses. Additionally, the number of studies included is limited, owing to the low number of studies examining both the GM and temperament. These factors limit the generalizability of results.

This review had some limitations. First, data searches were conducted only on one major scientific database (PubMed) by a single author. Additionally, the citations of included papers were not scanned for potentially relevant papers. Therefore, some relevant articles may have been left out of the scope of this review. This is highlighted by the discrepancies in the included papers between this study and another recent systematic literature review (Alving-Jessep et al., 2022).

5.5 Conclusion

The primary objective of this review was to determine whether there was evidence of an association between GM composition and diversity and early childhood temperament. Some interesting patterns could be seen emerging, regarding the possible role of *Bifidobacteriaceae* in promoting pro-social behavior, as well as the butyrate-producing families of *Lachnospiraceae*, *Ruminococcaceae* and *Dialister*, all members of the phylum Bacillota (Firmicutes) having an association with Positive/Negative affectivity and Regulation. Although these observations find support in the literature, they can, however, only be considered tentative and evidence on the subject remains preliminary owing mainly to the limited number of studies available as well as the observed significant heterogeneity in methodology and study populations. A more uniform control of covariables would increase the comparability and reliability of results and a larger scope of different populations as well as larger sample sizes would increase generalizability. Further research is needed both to replicate existing results across different populations and to expand the scope of research to include functional aspects of the GM as well as a longitudinal approach. This would precipitate identification of possible early life markers of later behavioral and mental health problems, providing a possibility for developing interventions and prevention measures targeted at modifiable early life antecedents.

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